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ARTICLES

Ultra HPLC Method for Fixed Dose Combination of Azilsartan Medoxomil and Chlorthalidone: Identification and *in silico* Toxicity Prediction of Degradation Products[1]

Gananadhamu Samanthula[a], *, Debasish Swain[a], Gayatri Sahu[b], Shweta Bhagat[c], and P. V. Bharatam[c]

[a] *National Institute of Pharmaceutical Education and Research (NIPER), Balanagar, Hyderabad, Telangana, India*

[b] *United States Pharmacopeia (USP) India Pvt. Ltd., Hyderabad, Telangana, India*

[c] *National Institute of Pharmaceutical Education and Research (NIPER), SAS Nagar, Mohali, Punjab, India*

**e-mail: gana@niperhyd.ac.in*

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Abstract—The fixed dose combination of azilsartan medoxomil potassium and chlorthalidone has been introduced for the effective treatment of hypertension. In the present work a rapid, simple and accurate stability indicating ultra HPLC assay method has been developed. The separation of azilsartan medoxomil, chlorthalidone and their degradation products were accomplished on an Acquity UPLC BEH C18 (100 mm × 2.1 mm, 1.7 μm) column using mobile phase combination of 0.02% trifluoroacetic acid in water and acetonitrile in gradient mode. The forced degradation products were identified using liquid chromatography–electrospray ionisation-quadrupole time of flight-tandem mass spectrometry (**LC–ESI-QTOF–MS/MS**) and accurate mass experiments. The *in silico* toxicities of the degradation products for both the drugs were evaluated. The proposed method was validated as per the ICH Q2 (R1) guideline for selectivity, linearity, precision, accuracy and robustness.

Keywords: azilsartan medoxomil potassium, chlorthalidone, ultra HPLC, LC–ESI-QTOF–MS/MS, *in silico* toxicity

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Azilsartan medoxomil potassium (**AZP**) is chemically known as 1-[{2'-(2,5-dihydro-5-oxo-1,2,4-oxadiazol-3-yl)[1,1'-biphenyl]-4-yl]methyl]-2-ethoxy-1H-benzimidazole-7-carboxylic acid-(5-methyl-2-oxo-1,3-dioxol-4-yl) methyl ester potassium [1]. It is an angiotensin receptor blocker (ARB) used in the treatment of hypertension and has more efficacy than the other "sartans" commonly used [2, 3]. Chlorthalidone (**CLD**) is chemically 2-chloro-5-[(1RS)-1-hydroxy-3-oxo-2,3-dihydro-1H-isoindol-1-yl] benzene sulphonamide. It is a long acting diuretic drug which enhances the rate of urination and leads to outflow of Na^+ and K^+ ions from the body. The diuretics are used for the treatment of hypertension, either alone or in combination with sartans [4]. The combination of AZP and CLD showed a greater degree of safety and efficacy than the other combinations [5]. The management of hypertension is a lifelong process where the medication has to be continued by the patients for a long duration. Hence the quality of drugs used in the treatment is of paramount importance. Quality of drugs depends on the purity and safety aspects of the drugs. Purity of the drug is achieved by controlling the impurities and degradation products. Thorough forced degradation studies during development of stability indicating assay method facilitate the identification of the degradation products which can then be evaluated for the possible biological implications [6, 7]. The *in silico* tools provide a quick and non tedious platform to predict toxicities associated with the degradation products and impurities. They give a preliminary indication of the pharmacological implications of the impurities which can then be useful for the appropriate changes to be made in the synthetic procedure or formulations, as the case may be [8].

The fixed dose combination of two or more drugs is common in the present day medical treatment. Thus developing a single precise method capable of separating and estimating both the components in presence of their impurities and degradation products is advantageous and time saving. The literature showed a few analytical methods for the simultaneous estimation of AZP and CLD in the recent past. An HPLC method was proposed to estimate both the drugs [9]. We recently reported the characterization of degradation products of AZP and LC–MS compatible stability

[1] The article is published in the original.

Table 1. Gradient program for elution of AZP and CLD and degradation products at a flowrate of 0.3 mL/min

Time, min	% A (0.02% trifluoroacetic acid)	% B (acetonitrile)
Initial	80	20
1.0	80	20
4.0	25	75
6.0	25	75
6.5	80	20
9.0	80	20

indicating assay method for AZP [10, 11]. But no analytical methods are available for simultaneous separation and identification of forced degradation products of AZP and CLD combination. In the present work the drugs, AZP and CLD were subjected to forced degradation and the resulting degradation products were separated by using a reversed-phase ultra HPLC (**UHPLC**) method. The structures of the degradation products were identified using LC–ESI-QTOF–MS/MS and accurate mass measurements. The *in silico* toxicity of the degradation products were evaluated using TOPKAT software.

EXPERIMENTAL

Chemicals and reagents. AZP and CLD were obtained as gratis samples from Mylan Laboratories (Hyderabad, India) and Symed Labs (Hyderabad, India), respectively. HPLC grade acetonitrile was obtained from Merck (Darmsdat, Germany) and trifluoroacetic acid (**TFA**) was supplied by Qualigens Fine Chemicals (Mumbai, India). AR grade hydrochloric acid, sodium hydroxide and hydrogen peroxide (30%, w/v) were obtained from SD Fine Chem Pvt. Ltd. The Nylon membrane filter (0.2 μm) manufactured by Advanced Microdevices (Ambala, India) was used to filter mobile phase and sample solutions. Milli-Q water obtained from Millipore water system (Milford, MA, USA) was used in making solutions. A composition of water and acetonitrile in the ratio of 50 : 50 (v/v) was used as the diluent. This diluent was used to prepare all standard and sample solutions.

Instrumentation. The chromatography was performed on an Acquity UPLC system (Waters, Milford, MA), equipped with a binary solvent manager, sample manager and a photodiode array (**PDA**) detector. UHPLC–MS instrument consisting of Agilent Infinity 1200 series coupled with a PDA detector and a quadrupole time-of-flight mass spectrometer (QTOF LC/MS 6540 series, Agilent Technologies, USA) equipped with an electrospray ionization source was used for the MS analysis. The thermal degradation studies were carried out in Osworld laboratory oven (Osworld Scientific Pvt. Ltd., India). A photostability chamber (Osworld OPSH-G-16 GMP series, Osworld Scientific Equipments Pvt. Ltd., India) was set at 40 ± 5°C and relative humidity (RH) of 75 ± 5%. This photostability chamber has ultraviolet and fluorescent lamps, as recommended by ICH Q1B guideline [12].

Ultra HPLC conditions. Aquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 μm) was used as stationary phase. The mobile phase consisted of 0.02% (v/v) TFA in water and acetonitrile. The separation of AZP, CLD and degradation products was achieved by gradient elution (Table 1) at a flow rate of 0.3 mL/min. The column oven temperature was maintained at 25°C. The sample injection volume was 2 μL. The chromatograms were integrated at detection wavelength of 225 nm. The UHPLC system control, data collection and data processing were accomplished using Waters Empower 3 software.

Mass spectrometric conditions. The drugs AZP and CLD are basic in nature and thus positive ionization mode was selected for recording the MS spectra. The fragmentor voltage was optimized based on the extent of fragmentation shown by the drug molecules in study. Thus, after performing number of systematic trials the fragmentor voltage was set at 144 V. The capillary voltage, which is generally molecular weight dependent parameter, was set at 3500 V for AZM and CLD wheras skimmer was set at 65 V. Nitrogen gas was used as the drying gas and its flow rate and temperature are responsible for the molecule to ionize efficiently. Therefore, the drying was set at 325°C and flow rate of 10 L/min. The nebulizing gas set at 40 psi was optimized based on the LC-flow into the mass and also dependent on the mobile phase composition. The MS/MS experiments were carried out using the collision-induced dissociation with nitrogen as collision gas. The mass spectrometric data acquisitions were under the control of Mass Hunter software.

Forced degradation studies. Forced degradation studies of AZP and CLD were carried out under hydrolytic (acid, base and neutral), oxidative, photolytic and thermal stress conditions as per ICH Q1A (R2) guideline [13]. AZP and CLD at a concentration of 1 mg/mL each were used to study all the solution state forced degradation studies.

Hydrolysis. Acidic hydrolysis was carried out for AZP and CLD in 0.1 M HCl for 1 h and 5 M HCl for 4 h at 60°C, respectively. The basic hydrolysis was performed at 1 M NaOH at 60°C for 24 h for AZP, whereas for CLD in 1 M NaOH at 60°C for 40 min. In neutral hydrolysis water was added to the drug samples (AZP and CLD separately) and exposed to 60°C for 1 h.

Oxidative stress. Oxidative studies were carried out at 0.5% H_2O_2 at room temperature for 3 h where AZP showed optimum degradation.

Thermal stress. AZP and CLD powder was spread as a layer of about 1 mm thickness in a Petri dish separately and kept in dry air oven at 100°C for 48 h.

Photolytic stress. For photolytic degradation the solid samples were exposed to UV light at an irradiation dose of 200 W h/m^2 and fluorescent light at 1.2 million lux h in separate Petri dishes. Control samples were also prepared by wrapping the Petri dishes with aluminium foil prior to holding them under similar conditions to those of the stressed samples. The solution state stability under UV light and fluorescent light was studied by dissolving the drug in the diluent and then placing it in the photolytic chamber.

On completion of the forced degradation studies, the samples (AZP and CLD) of acid and base hydrolysis were neutralised before injecting into the chromatograph. All the samples were diluted to contain 40 μg/mL of AZP and 30 μg/mL of CLD before injecting to the chromatographic system

***In silico* toxicity.** A number of software tools are available for toxicity prediction, covering a range of toxicity endpoints. To assess the toxicity of the drug and its degradation products, expert systems like TOPKAT (Discovery Studio 2.5, Accelrys, Inc., San Diego, CA, USA) was used. TOPKAT (Toxicity Prediction by Komputer Assisted Technology) estimates the toxicity of a compound quantitatively. By using well established Quantitative Structure Toxicity Relationship (QSTR) models, TOPKAT predicts toxicological end points in term of probability values. Probability values from 0.0 to 0.30 are considered as low probabilities for any toxicological end point whereas probability values greater than 0.70 are considered as high probabilities.

Method validation. *Selectivity.* The ICH Q1A (R2) guideline recommends conducting forced degradation studies on the drug substance to establish its inherent stability characteristics to demonstrate the selectivity and stability-indicating capability of the proposed method. The forced degradation samples were used for proving the selectivity of the method [14].

Linearity. The linearity was demonstrated from 80 to 120% of standard concentration of each drug. Each standard solution of AZP at a concentration of 32, 36, 40, 44 and 48 μg/mL was analyzed in triplicate for establishing the linearity. CLD is available either as 12.5 mg or 25 mg in combination with 40 mg of AZP. Hence the linearity for CLD was evaluated in a broad range by analyzing standard solutions of 10, 15, 20, 25 and 30 μg/mL in triplicate. The linear regression of the calibration curve (peak area of standard substances plotted against respective concentrations) and correlation coefficient were used for data evaluation.

Precision. The precision of the proposed method was investigated by analysing six sample preparations at 100% nominal concentration. Intermediate precision was studied using different equipment, performing the analysis on a different day and by different analysts.

Accuracy. The accuracy of the proposed method was evaluated by spiking known concentrations of the standard drug into the synthetic mixture of the excipients and calculating the mean recoveries. The accuracy was performed at three different levels of concentration (80, 100 and 120%) of the assay concentrations. The mean recoveries and the RSD (%) were calculated.

Robustness. Robustness is an indication of the developed method to remain unaltered when small and deliberate changes have been made to the method parameters. The parameters like temperature of the column and flow rate of the mobile phase were altered and the observations were made.

Solution stability. The stability of the sample solution was established by storing the sample solution at ambient temperature. The sample solutions were analyzed at definite time periods over a time of 48 h and the concentration was determined and compared against a fresh sample solution.

System suitability. System suitability parameters were developed based on the data obtained from method development and validation studies. The precision of retention time and peak areas, resolution between critical peak pairs, tailing and theoretical plate number (efficiency) for AZP and CLD were considered as system suitability parameters.

RESULTS AND DISCUSSION

Method development and optimization. The primary agenda behind developing an UHPLC method in the present study was to estimate both AZP and CLD in a single run in the presence of their degradation products. CLD is a polar molecule where as AZP is relatively non-polar molecule. Hence the method development for separation of CLD and AZP was quite challenging. Among the different columns screened, Acquity UPLC BEH C18 (100 × 2.1 mm, 1.7 μm) column was selected as stationary phase as it showed the better retention of both the drugs. The aqueous component of the mobile phase was finalized based on a number of preliminary trials which included ammonium formate (at pH 3.0, 4.0), ammonium acetate (at pH 5.0, 5.5 and 6.0), acetic acid, formic acid and TFA. Good peak shapes were obtained when TFA was used. The final strength of the TFA solution was optimized as 0.02% in water. The organic solvent composition in the mobile phase was selected based on different proportions of methanol and acetonitrile. Methanol produced broad peak of AZP and CLD. Also the column pressures were found to be high when methanol was used as the mobile phase. Acetonitrile produced good sharp peaks with good retention

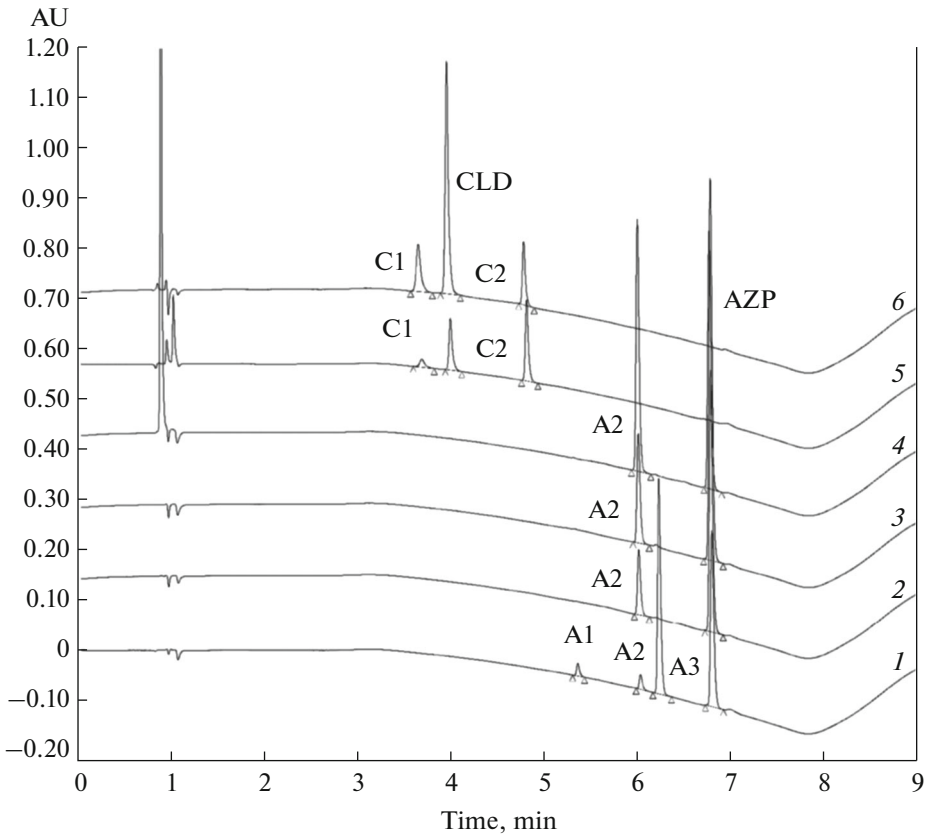


Fig. 1. Overlaid chromatograms of acid degradation of AZP (*1*), base degradation of AZP (*2*), neutral degradation of AZP (*3*), oxidative degradation of AZP (*4*), acid degradation of CLD (*5*), base degradation of CLD (*6*).

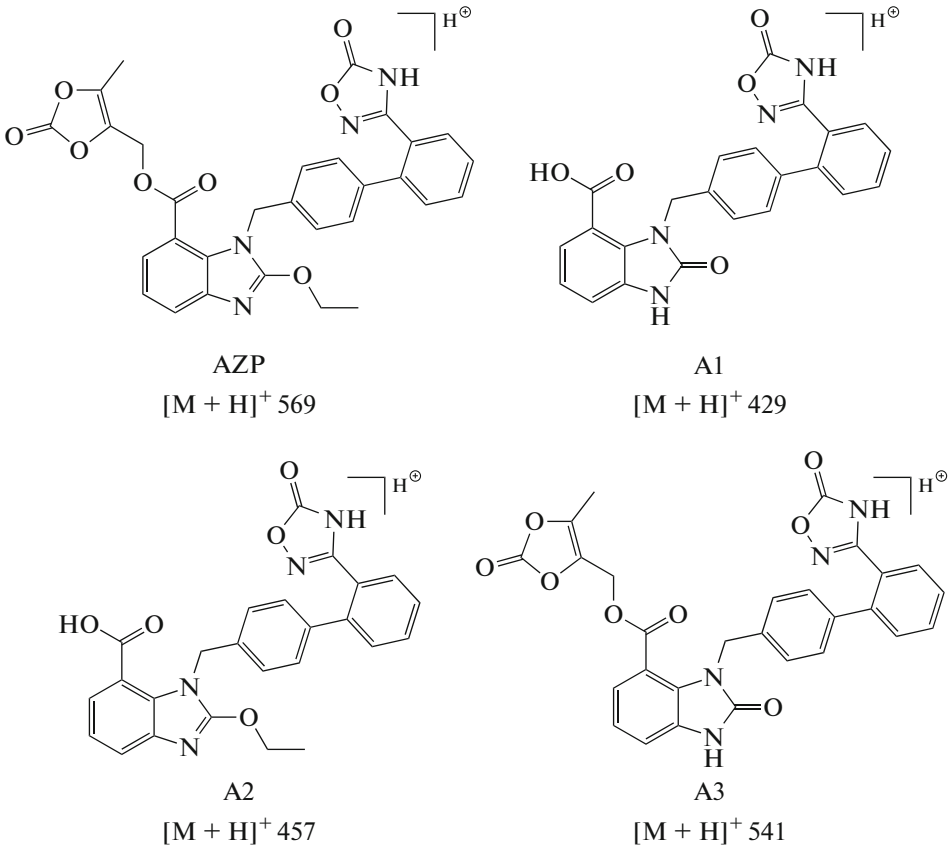
and optimum column pressure. The flow rate of mobile phase was optimized at 0.3 mL/min. Injection volume was fixed at 2 μL and assay concentrations of AZP and CLD were set at 40 and 12.5 μg/mL, respectively, as they enabled the detection of drugs and their degradation products. The wavelength of analysis was selected to be 225 nm where both the drugs and degradation products showed good UV light absorption. The developed method showed good resolution among the peaks of drug and the degradation products. The chromatographic separation of AZP, CLD and their degradation products is shown in Fig. 1. Separate chromatograms for AZM and CLD were included to clearly show the degradation behaviour of individual drugs at various degradation conditions. The proposed method was also transferred to LC–MS for characterisation of the degradation products. Electrospray ionisation (ESI) with positive ionization mode had provided the sensitive detection of AZP, CLD and their degradation products.

LC–MS/MS study of degradation products. A thorough forced degradation study was carried out and the samples from the degradation studies were subjected to mass spectrometric analysis in order to characterise the structure of degradation products. The structures for the degradation products were allocated based on the accurate mass measurements for the parent and the product ions.

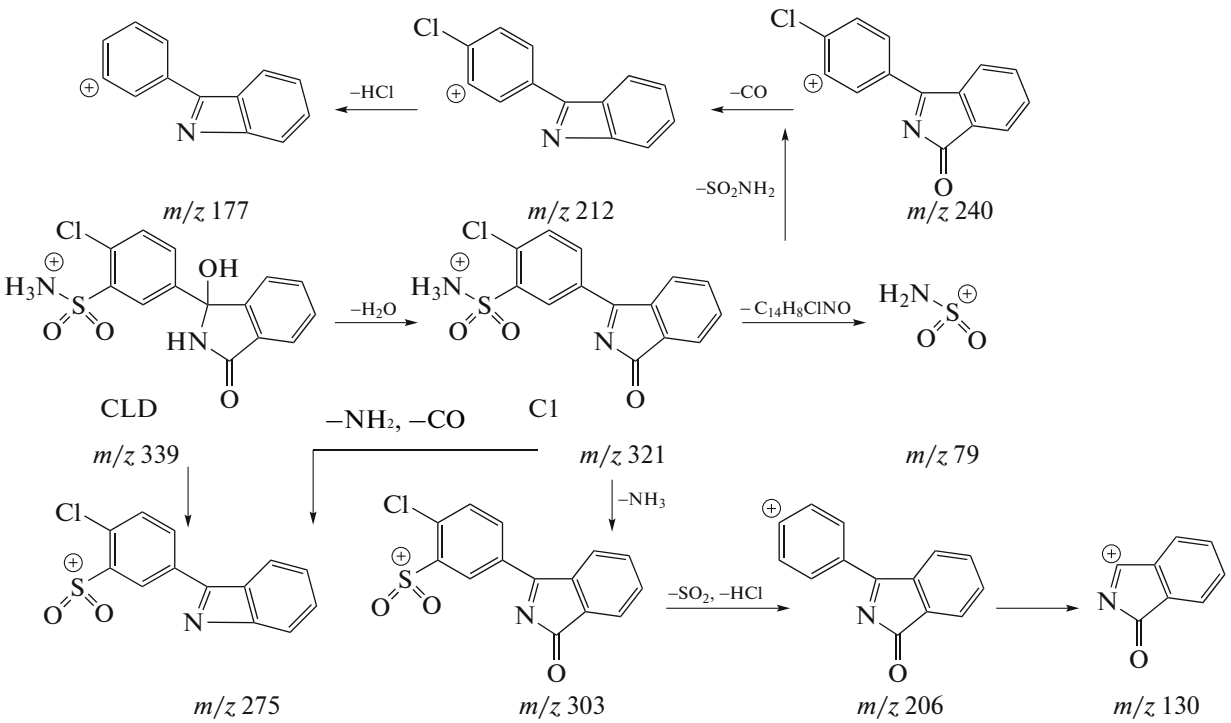
AZP and its degradation products. Recently we reported the degradation products of AZP (Swain et al., 2015a and Swain et al., 2015b). The degradation products of AZP were identified by comparing accurate mass data with the reported methods. The chemical structures of AZP and its degradation are shown in Scheme 1.

CLD and its degradation products. *CLD.* The CID spectrum of protonated CLD (Fig. 2, curve *1*): $[M + H]^+$, *m/z* 339) showed product ions of *m/z* 321 (loss of H_2O from the *m/z* 339), *m/z* 303 (loss of NH_3 from the *m/z* 321), *m/z* 240 (loss of SO_2NH_2), *m/z* 212 (loss of CO from *m/z* 240) and *m/z* 177 (loss of HCl from *m/z* 212) (Scheme 2).

C1 (Fig. 2, curve *2*): $[M + H]^+$, *m/z* 321) showed product ions of *m/z* 303 (loss of NH_3 from the *m/z* 321), *m/z* 240 (loss of SO_2NH_2), *m/z* 212 (loss of CO from *m/z* 240), *m/z* 206 (loss of SO_2 and HCl from *m/z* 303), *m/z* 177 (loss of HCl from *m/z* 212), *m/z* 130 (loss of C_6H_4 from *m/z* 206) and *m/z* 79 (loss of $C_{14}H_8ClNO$ from *m/z* 321) (Scheme 2).



Scheme 1. Chemical structures of AZP and its degradation products.



Scheme 2. Mass spectral fragmentation of CLD and C1.

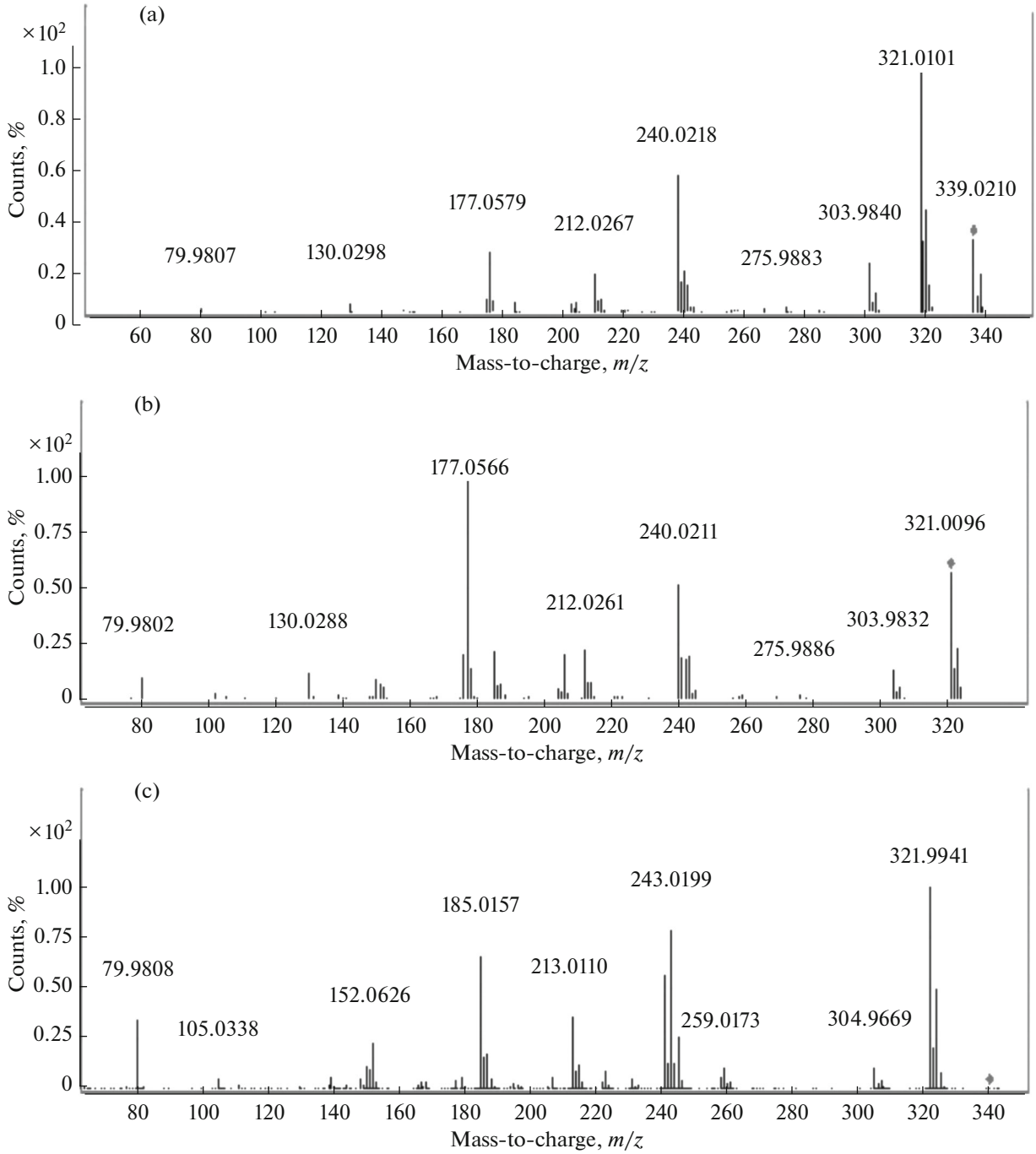
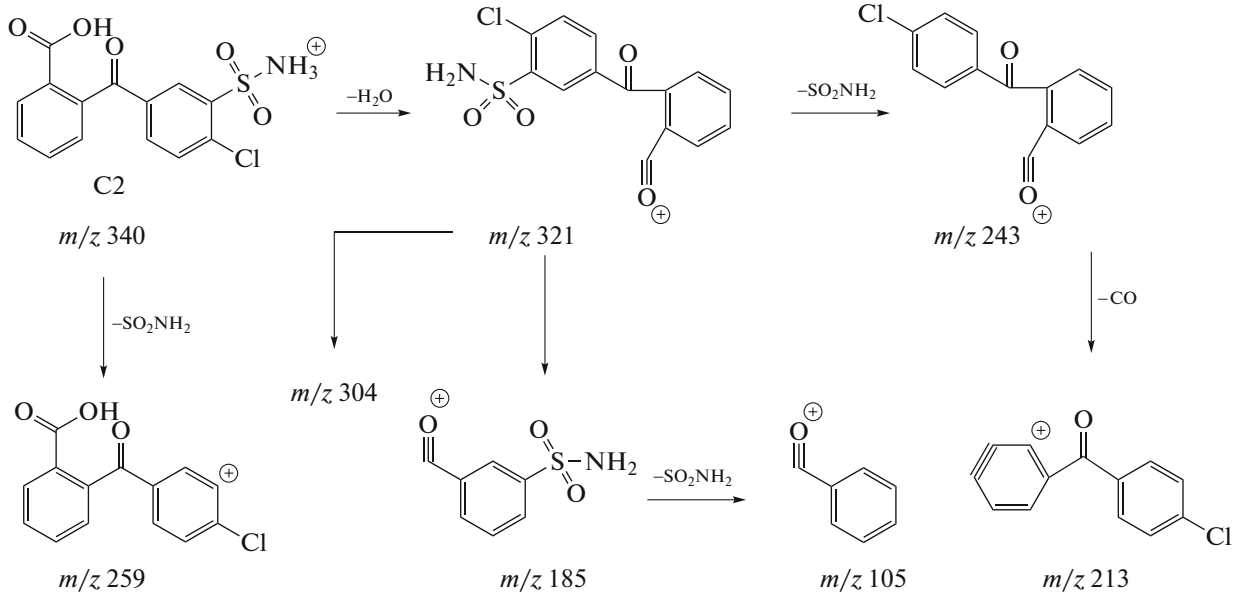


Fig. 2. MS/MS spectra of CLD (a), C1 (b), C2 (c).

C2 (Fig. 2, curve *3*): $[M + H]^+$, *m/z* 340) formed under hydrolytic conditions of stress showed product ions of *m/z* 321 (loss of H_2O from *m/z* 340), *m/z* 304 (loss of NH_3 from *m/z* 321), *m/z* 259 (loss of SO_2NH_2 from *m/z* 340), *m/z* 243 (loss of SO_2NH_2 from *m/z* 321), *m/z* 223 (loss of HCl from *m/z* 259), *m/z* 213 (loss of CH_2O from *m/z* 243), *m/z* 185 (loss of C_7H_2ClO from *m/z* 321), *m/z* 179 (loss of H_2Cl from *m/z* 213) and *m/z* 79 (loss of $C_{14}H_8ClNO$ from *m/z* 340) (Scheme 3). The chemical structures of CLD and its degradation products are shown in Scheme 4.

Table 2. Toxicity prediction data of AZP and its degradation products by TOPKAT

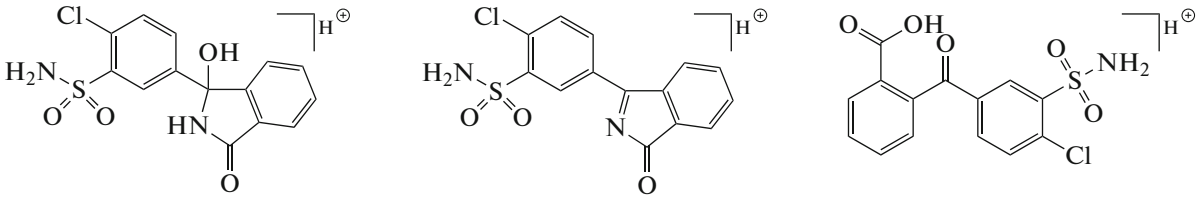
Model	AZP	A1	A2	A3
NTP carcinogenicity call (male rat) (v3.2)	1.000	1.000	1.000	1.000
NTP carcinogenicity call (female rat) (v3.2)	0.004	0.282	1.000	0.001
NTP carcinogenicity call (male mouse) (v3.2)	1.000	1.000	1.000	1.000
NTP carcinogenicity call (female Mouse) (v3.2)	0.004	0.451	0.119	0.061
FDA carcinogenicity male rat non vs. carc (v3.1)	0.000	0.000	0.000	0.000
FDA carcinogenicity male rat single vs. mult (v3.1)	0.000	0.000	0.000	0.000
FDA carcinogenicity female rat non vs. carc (v3.1)	0.000	0.000	0.000	0.000
FDA carcinogenicity female rat single vs. mult (v3.1)	0.000	0.000	0.117	0.000
FDA carcinogenicity male mouse non vs carc (v3.1)	0.001	0.000	1.000	0.000
FDA carcinogenicity male mouse single vs. mult (v3.1)	0.000	0.460	0.000	0.008
FDA carcinogenicity female mouse non vs. carc (v3.1)	0.000	0.000	0.000	0.000
FDA carcinogenicity female mouse single vs. mult (v3.1)	0.000	0.000	0.000	0.000
Weight of evidence carcinogenicity call (v5.1)	1.000	0.977	0.996	1.000
Ames mutagenicity (v3.1)	0.146	0.000	0.000	0.000
Developmental toxicity potential (DTP) (v3.1)	0.000	0.983	0.000	0.000
Rat oral LD50 (v3.1) (mg/kg)	13.00	10.000	282	10.000
Rat maximum tolerated dose – feed/water (v6.1) (μg/kg)	2.3×10^3	5.8×10^3	937	15×10^3
Rat inhalational LC50 (v6.1) (mg/m^3/H)	127×10^{-3}	457	9.7×10^{-3}	10×10^3
Chronic LOAEL (v3.1) (mg/kg)	2.6	1.7×10^3	41.7	104
Skin irritation (v6.1)	0.000	0.000	0.002	0.000
Skin sensitization NEG vs. SENS (v6.1)	1.000	1.000	1.000	1.000
Skin sensitization MLD/MOD vs. SEV (v6.1)	0.000	0.000	0.000	0.000
Ocular irritancy SEV/MOD vs. MLD/NON (v5.1)	1.000	1.000	1.000	1.000
Ocular irritancy SEV vs. MOD (v5.1)	0.000	0.000	0.000	0.000
Ocular irritancy MLD vs. NON (v5.1)	0.999	1.000	0.998	0.996
Aerobic biodegradability (v6.1)	0.000	0.000	0.000	0.000
Daphnia EC50 (v3.1) (mg/L)	5.3	0.127	0.275	0.140



Scheme 3. Mass spectral fragmentation of C2.

Table 3. Toxicity prediction data of CLD and its degradation products by TOPKAT

Model	CLD	C1	C2
NTP carcinogenicity call (male rat) (v3.2)	0.001	0.000	1.000
NTP carcinogenicity call (female rat) (v3.2)	0.001	0.000	0.000
NTP carcinogenicity call (male mouse) (v3.2)	0.000	0.000	1.000
NTP carcinogenicity call (female mouse) (v3.2)	1.000	1.000	0.425
FDA carcinogenicity male rat non vs. carc (v3.1)	1.000	0.999	0.000
FDA carcinogenicity male rat single vs. mult (v3.1)	0.900	0.000	0.000
FDA carcinogenicity female rat non vs. carc (v3.1)	0.010	0.000	0.000
FDA carcinogenicity female rat single vs. mult (v3.1)	0.000	0.000	0.000
FDA carcinogenicity male mouse non vs. carc (v3.1)	0.997	0.394	1.000
FDA carcinogenicity male mouse single vs. mult (v3.1)	1.000	0.000	0.000
FDA carcinogenicity female mouse non vs. carc (v3.1)	0.126	0.252	0.006
FDA carcinogenicity female mouse single vs. mult (v3.1)	0.000	0.000	0.000
Weight of evidence carcinogenicity call (v5.1)	0.000	0.000	1.000
Ames mutagenicity (v3.1)	0.012	0.000	0.000
Developmental toxicity potential (DTP) (v3.1)	0.999	0.002	0.992
Rat oral LD_{50} (v3.1) (mg/kg)	5.7	2,600	10,000
Rat maximum tolerated dose – feed/water (v6.1) (μg/kg)	52×10^3	1.3×10^6	6.2
Rat inhalational LC_{50} (v6.1) (mg/m^3/H)	25.7	2.4×10^3	3.8
Chronic LOAEL (v3.1) (mg/kg)	23.7	163	1.8
Skin irritation (v6.1)	1.000	0.003	1.000
Skin sensitization NEG v SENS (v6.1)	0.999	0.998	1.000
Skin sensitization MLD/MOD v SEV (v6.1)	0.282	1.000	1.000
Ocular irritancy SEV/MOD vs. MLD/NON (v5.1)	0.254	0.000	0.000
Ocular irritancy SEV vs. MOD (v5.1)	0.000	0.001	0.041
Ocular irritancy MLD vs. NON (v5.1)	0.796	0.841	1.000
Aerobic biodegradability (v6.1)	0.566	0.000	0.000
Daphnia EC_{50} (v3.1) (mg/L)	2.1	1.7	1.9



CLD $[M + H]^+$ 339 C1 $[M + H]^+$ 321 C2 $[M + H]^+$ 340

Scheme 4. Chemical structures of CLD and its degradation products.

***In silico* toxicity.** The toxicities of the drugs and degradation products were predicted using the TOPKAT software. It was observed that AZP, A1, A2, A3 and C2 showed higher probability of NTP carcinogenicity in male rat and male mouse, weight of evidence carcinogenicity and ocular irritancy. Both the drugs and all the degradation products were found to have higher possibility for skin sensitization. A1, CLD and C2 also showed the probability for the occurrence of developmental toxicity potential, whereas A2, CLD and C2 indicated the presence for FDA carcinogenicity male mouse non vs. carc test. All the toxicities are shown in Tables 2 and 3.

Method validation. After optimizing the method, validation of the same was performed as per ICH guidelines (ICH, 2005). The method was validated

Table 4. Linearity data for the simultaneous determination of AZP and CLD

Parameter	AZP	CLD
Calibration range, μg/mL	32–48	10–30
Correlation coefficient	0.9998	0.9997
Slope	2.98×10^4	3.06×10^4
Intercept	3.8×10^4	-5.3×10^3
SD of slope	5.3×10^2	2.9×10^2
SD of intercept	2.1×10^4	6.2×10^3

Table 5. Precision data for the simultaneous determination of AZP and CLD

Precision parameter	RSD, %	
	AZP	CLD
Method precision	0.15	0.18
Intra-day precision	0.12	0.11
Inter-day precision	0.37	0.13
Analyst I	0.10	0.10
Analyst II	0.40	0.10
Instrument I	0.13	0.15
Instrument II	0.23	0.21

according to a standard procedure and all the parameters of the validation process were evaluated to demonstrate the suitability of the method.

Selectivity. The specificity of the method was established by evaluating the peak purity of individual drugs and their degradation products under various stress conditions. The purity angle was found to be lower than the purity threshold which indicates that the peaks were pure under all the conditions.

Linearity. The developed method showed a linear response in the concentration range of 80–120% of the assay concentration of individual drugs. The linear regression equation obtained for AZM was $y = 29794x + 37628$ and for CLD $y = 30649x - 5340$. The correlation coefficient of both drugs was calculated and found to be greater than 0.999 (Table 4).

Precision. The precision of the method was evaluated by analyzing duplicates of six standard preparations. The intermediate precision was also carried out on different days, different column and different instrument (Table 5). The low RSD indicated the high precision of the developed method.

Accuracy. The accuracy of the developed method was established at three different levels (80, 100 and 120%) of the assay concentrations of the drugs. The known quantity of drug was spiked into the synthetic mixture prepared using different excipients consisting of mannitol (38%, w/w), microcrystalline cellulose

Table 6. Accuracy data for the simultaneous determination of AZP and CLD

Amount added, mg	Amount found, mg	Recovery, %	RSD, %
AZP			
32.05	32.09	100.12	
32.09	32.01	99.75	0.72
32.12	32.49	101.15	
40.05	40.10	100.12	
40.08	40.28	100.50	0.20
40.11	40.19	100.20	
48.77	48.80	100.06	
48.82	48.76	99.88	0.09
48.91	48.88	99.94	
CLD			
10.08	10.12	100.40	
10.06	10.08	100.20	0.15
10.10	10.11	100.10	
20.05	20.02	99.85	
20.09	20.05	99.80	0.05
20.12	20.10	99.90	
30.05	30.08	100.10	
30.11	30.15	100.13	0.20
30.09	30.02	100.07	

(7.5%, w/w), hydroxypropylcellulose (3%, w/w), crosspovidone (2%, w/w), magnesium stearate (1.5%, w/w), hydroxypropylmethyl cellulose (2%, w/w), talc (7.5%, w/w), titanium dioxide (1.5%, w/w), ferric oxide red (1%, w/w) and polyethylene glycol (1.5%, w/w) which are commonly used in formulations. The mean recoveries of the drugs were calculated and the recovery was found to be ±2% of the quantity added (Table 6).

Robustness. The robustness studies were carried out by deliberately altering the temperature of the column (25 ± 5°C) and the flow rate of the mobile phase (0.30 ± 0.05 mL/min), and the samples were analyzed. The system suitability parameters under the altered conditions were passed. Hence the proposed method is robust.

Solution stability. The solutions prepared for the analyses were stable for a period over 48 h. The stability was established by comparing the sample with the freshly prepared solutions.

System suitability. The RSD of retention time and area of six replicate injections of the standard solution of AZP and CLD was set below 1.0%. The resolution between the critical pairs C1/CLD and A2/A3 was fixed at greater than 2.0. Peak tailing of less than 1.5 and theoretical plates more than 5000 were proposed as system suitability parameters.

CONCLUSIONS

A novel UHPLC method was successfully developed and validated for the simultaneous determination of AZP and CLD. The total analysis time was 9 min, within which both drugs and their degradation products were separated from each other thereby showing the stability indicating power of the method. The *in silico* toxicities for the degradation products of AZP and CLD were evaluated using the TOPKAT software. Validation results indicated the method to be selective, precise, linear and accurate.

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REFERENCES

1. Baker, W.L. and White, W.B., *Ann. Pharmacother.*, 2011, vol. 45, no. 12, p. 1506.
2. Lam, S., Cardiol. Rev., 2011, vol. 19, no. 6, p. 300.
3. Sica, D., White, W.B., Weber, M.A., Bakris, G.L., Perez, A., Cao, C., Handley, A., and Kupfer, S., *J. Clin. Hypertens.*, 2011, vol. 13, no. 7, p. 467.
4. Ernst, M.E., Carter, B.L., Goerdt, C.J., Steffensmeier, J.J., Phillips, B.B., Zimmerman, M.B., and Bergus, G.R., *Hypertension*, 2006, vol. 47, no. 3, p. 352.
5. Cushman, W.C., Bakris, G.L., White, W.B., Weber, M.A., Sica, D., Roberts, A., Lloyd, E., and Kupfer, S., *Hypertension*, 2012, vol. 60, no. 2, p. 310.
6. Reynolds, D.W., Facchine, K.L., Mullaney, J.F., Alsante, K.M., Hatajik, T.D., and Motto, M.G., *Pharm. Technol.*, 2002, vol. 26, p. 48.
7. Wegienka, L.C. and Weller, J.M., *Arch. Int. Med.*, 1964, vol. 114, no. 2, p. 232.
8. Blessy, M., Patel, R.D., Prajapati, P.N., and Agrawal, Y., *J. Pharm. Anal.*, 2014, vol. 4, no. 3, p. 159.
9. Sravani, P., Rubesh, S., Duganath, N., and Devanna, N., *Int. J. Pharma Sci.*, 2014, vol. 4, no. 5, p. 725.
10. Swain, D., Patel, P.N., Palaniappan, I., Sahu, G., and Samanthula, G., *Rapid Commun. Mass Spectrom.*, 2015, vol. 29, no. 15, p. 1437.
11. Swain, D., Sahu, G., and Samanthula, G., *J. Anal. Bioanal. Tech.*, 2015, vol. 6, no. 4, 254. doi 10.4172/2155-9872.1000254
12. *Q1B Stability Testing: Photostability Testing of New Drug Substances and Products*, Proc. Int. Conf. Harmonization, Geneva, 1996.
13. *Q1A(R2) Stability Testing of New Drug Substances and Products*, Proc. Int. Conf. Harmonization, Geneva, 2003.
14. *Q2 (R1) Validation of Analytical Procedures*, Proc. Int. Conf. Harmonization, Geneva, 2005.